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Original Article

MITOCHONDRIAL DNA CHARACTERIZATION OF HIGH ROYAL JELLY-PRODUCING HONEYBEES (HYMENOPTERA: APIDAE) IN CHINA

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Abstract

China is the largest producer and exporter of royal jelly in the world. The high production of royal jelly in China is mainly attributed to a high royal jelly-producing lineage of honeybees (*Apis mellifera*) (HRJB). However, few studies have been conducted on the genetic characterization of HRJB. In this study, the mitochondrial DNA intergenic region between cytochrome oxidase I and II (COI-COII) and the mitochondrial NADH dehydrogenase subunit 2 sequences (ND2) were determined for 90 HRJB colonies, collected from the regions of China where HRJB originated, and 25 unimproved *A. m. ligustica* colonies from China. COI-COII sequence analysis revealed two mitotypes (C1 and C2d) in HRJB colonies and one mitotype (C1) in unimproved *A. m. ligustica* colonies. The main mitotype (C1) in HRJB accounted for 93% of the colonies. Based on ND2 sequences, four and two mitotypes were found in HRJB and unimproved *A. m. ligustica* colonies, respectively. Sequence alignment showed that nucleotides in three positions of the ND2 sequence were different between the main mitotype of HRJB and that of unimproved *A. m. ligustica*. Our study suggested that HRJB was bred from *A. m. ligustica* and possibly had genetic characteristics different from unimproved *A. m. ligustica.*

Keywords: *A. m. ligustica*, COI-COII, HRJB, mitotype, mtDNA, ND2

INTRODUCTION

Royal jelly is an important functional food in many countries (Ramadan & Al-Ghamdi, 2012). China is the world's largest producer and exporter of royal jelly with an annual production of 3000–4000 tonnes (ASAC, 2015). The high production of royal jelly in China is mainly attributed to the development of a new lineage of *Apis mellifera* with high royal-jelly producing performance (high royal-jelly producing honeybees, HRJB) (Cao et al., 2016). From the 1960s to the 1980s, beekeepers in some regions of China selected *A. mellifera* colonies for high royal jelly production. After semi-controlled and directive breeding for decades, royal jelly production of some colonies was more than ten-fold higher than that of unimproved colonies (Cao et al., 2016). HRJB was recognized by the Chinese government in the late 1980s and was rapidly introduced to other areas of China, which resulted in a great increase in the production of royal jelly in China (CNCAGR, 2011).

It was believed that HRJB was bred from *A. m. ligustica* on the basis that *A. m. ligustica* was the first subspecies of *A. mellifera* introduced into China, as well as being the most widely-distributed subspecies (Yin et al., 2011; Cao et al., 2016). However, there was a lack of evidence, particularly molecular data, to support any relationship between HRJB and *A. m. ligustica.* Population genetics studies, using malate dehydrogenase locus or microsatellites, showed significant genetic differentiation between HRJB and other unimproved *A. m. ligustica* populations (Sun et al., 2004; Chen et al., 2005), which suggested

that HRJB might have different genetic characteristics from unimproved *A. m. ligustica*.

Methods based on the analysis of *COI-COII* sequences in mitochondrial DNA (mtDNA) have been widely used in the determination of honeybee lineages (Magnus, Tripodi, & Szalanski, 2011; Meixner et al., 2013; Coroian et al., 2014). Six evolutionary lineages (A, C, M, O, Y, and Z) have been found in *A. mellifera* according to morphometric or molecular studies (Ruttner, 1988; Franck et al., 2001; Alburaki et al., 2013). For example, mitotype C1 was found in all *A. m. ligustica* colonies as well as in some *A. m. carnica* (Carniolan honeybee) colonies (Franck et al., 2000a; Sušnik et al., 2004). Moreover, the mtDNA *ND2* sequence exhibits a high degree of genetic variability and has been widely used to discriminate different honeybee populations (Arias & Sheppard, 1996; Franck et al., 2000b). Therefore, in this paper, the mtDNA *COI-COII* and *ND2* sequences were determined for HRJB colonies and unimproved *A. m. ligustica* colonies in China to reveal the relationship between HRJB and *A. m. ligustica*, which will be important for the conservation and further improvement of HRJB.

MATERIAL AND METHODS

Sample collection

HRJB colonies from Pinghu, Xiaoshan and Changxing regions in the Zhejiang Province, China, where HRJB originated (Fig. 1), were sampled during 2012 and 2013. The three regions are about 100 km apart. Three HRJB colonies were sampled from each of ten breeders of honeybee queens from each region. Unimproved descendants of an *A. m. ligustica* population, imported into China at the beginning of the 20th century, have been raised for a long time in some areas of China. Different *A. m. ligustica* populations have also been introduced as breeding material into China in recent decades from Italy, the USA and Australia. All these *A. m. ligustica* populations have the standard yield of royal jelly and are preserved in the China National Genebank of

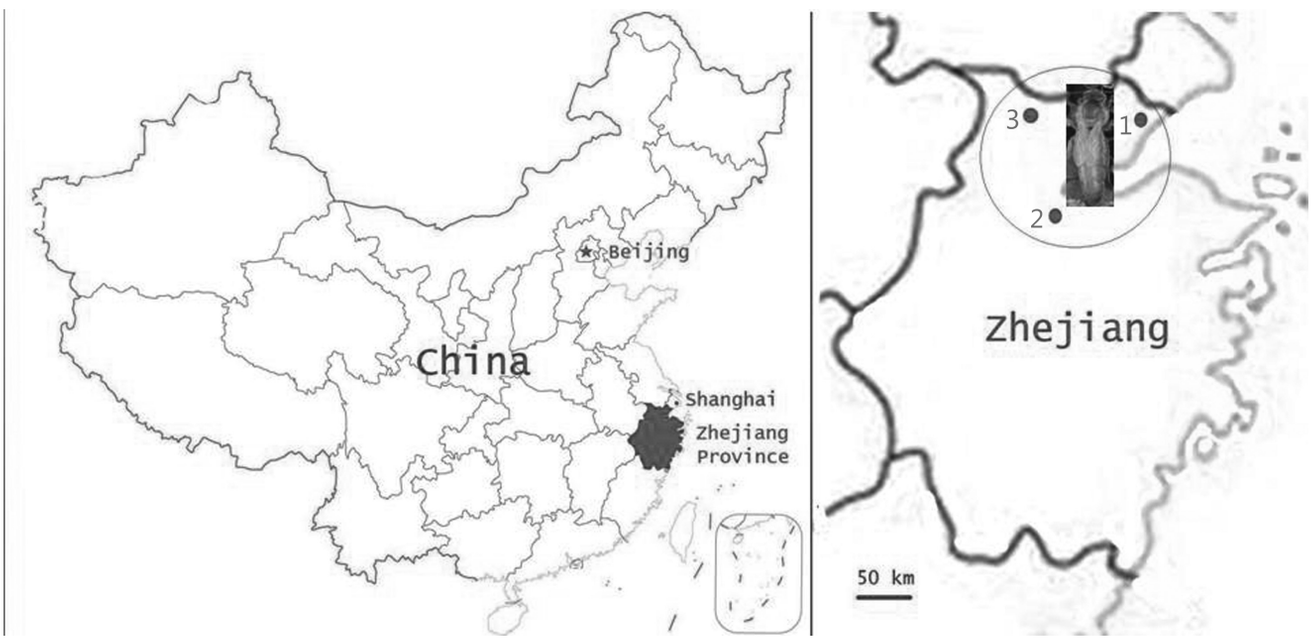

Fig. 1. The shaded area on the left-hand map indicates the main area of distribution of HRJB in China. The area bound by the circle on the right-hand map indicates the original area of distribution of HRJB. The dots denote the HRJB sample collection regions: Pinghu (1), Xiaoshan (2), and Changxing (3).

Honeybees (Jilin, China). Five colonies from each of the three recently introduced *A. m. ligustica* populations (from Italy, the USA and Australia, respectively) and ten colonies from the early introduced *A. m. ligustica* population (at the beginning of the 20th century) were collected at the same time for comparison with HRJB. Therefore, 90 HRJB colonies and 25 unimproved *A. m. ligustica* colonies were sampled in our study. Thirty adult honey bee workers were preserved in absolute ethanol and stored at -20°C until needed for analysis.

DNA extraction, amplification, and sequencing

Total DNA was isolated from the thorax of a single worker bee from each colony, using the standard three-step phenol-chloroform method (Sambrook, Fritsch, & Maniatis, 1989). The mtDNA *COI-COII* intergenic region was amplified by polymerase chain reaction (PCR), with primers E2 and H2, according to the protocol described by Cornuet, Garnery, & Solignac (1991). The mtDNA *ND2* sequence was amplified using the primers ILE and L2, as described by Arias et al. (1996). The PCR products were sequenced (Sangon Biotech Co. Ltd., Shanghai, China) in both directions with the primers used for amplification

Sequence alignment and analysis

DNA sequences were edited using LASERGENE 7.0, aligned by ClustalX 1.81 (Thompson et al., 1997). Mitotypes were first determined using DnaSP 5.10 (Librado & Rozas, 2009) and then aligned with sequences published in GenBank. Frequencies of mitotypes were calculated for the overall honey bee population and for the populations from each region. Sequence alignment of mitotypes was also performed by ClustalX 1.81.

RESULTS

Based on the *COI-COII* sequences, two mitotypes were found in HRJB colonies (GenBank accession numbers: MF136775 and MF136776). When aligned with sequences published in GenBank, MF136775 was identical to C1 (FJ478010, reported by Franck et al., 2001), while MF136776 was identical to C2d (FJ824584, reported by Muñoz et al., 2009). Mitotypes C1 and C2d had the following overall frequencies: 0.933 and 0.067, respectively (Tab. 1). Mitotype C1 accounted for 80% of the colonies from the Pinghu region, whereas the other six colonies belonged to mitotype C2d (present in colonies from four of the ten queen breeders sampled). All the colonies of HRJB honey bees from Xiaoshan and Changxing belonged to mitotype C1. All the unimproved *A. m. ligustica* colonies collected in China belonged to mitotype C1 (Tab. 1).

According to the *ND2* sequences, four mitotypes were found in all honeybee colonies (Tab. 2). They were designated as N1-N4 with the GenBank accession numbers MF136771-MF136774. In HRJB, N1 was the most common mitotype accounting for 80% of the colonies.

Table 1

Mitotypes and distributions based on COI-COII sequences

population	Region	Geographical Coordinates	Altitude	Number of Colonies	C1a Distribution	C2d Distribution
HRJB	Pinghu	30°45´N 121°07´E	~3 m	30	24	6
	Xiaoshan	30°07´N 120°24´E	~6 m	30	30	-
	Changxing	30°57´N 119°50´E	~200 m	30	30	-
A. m. ligustica	-	-	-	25	25	-

Table 2

Mitotypes and distributions based on *ND2* sequences

population	Region	Number of Colonies	N1 Distribution	N2 Distribution	N3 Distribution	N4 Distribution
HRJB	Pinghu	30	17	6	6	1
	Xiaoshan	30	29	-	-	1
	Changxing	30	26	-	-	4
A. m. ligustica	-	25	1	-	-	24

Table 3

Nucleotide differences among mitotypes of *ND2* sequences

Mitotype	Nucleotide Position (according to NC_001566)				
	504	541	685	999	1015
H1	C	C	A	T	C
H2	T	C	G	C	C
H3	C	C	A	T	T
NC	C	T	G	C	C

On the contrary, N4 was the main mitotype in unimproved *A. m. ligustica* colonies accounting for 96% of these colonies. In the HRJB colonies from the Pinghu region, mitotypes N2 and N3 were present at moderate frequencies (approximately 20% in each case). Sequence alignment was performed with the four mitotypes derived from the *ND2* sequences (nucleotide position according to NC_001566, reported by Crozier & Crozier, 1993) (Tab. 3). It showed that the nucleotides were different in position 541, 685 and 999 between the main mitotype N1 in HRJB colonies and the main mitotype N4 in unimproved non-HRJB *A. m. ligustica* colonies.

DISCUSSION

MtDNA sequences have previously been used to analyze molecular diversity and population genetic structures of honey bee populations from different regions, and have proved to be an effective tool (Franck et al., 2000b; Muñoz et al., 2009, 2012). This current study is the first genetic variation study, using primarily mtDNA sequences, conducted on HRJB honey bees from China.

Results of the *COI-COII* sequence analysis showed that the main mitotype C1 in HRJB was identical to that in unimproved *A. m. ligustica* colonies in China, which suggested that *A. m. ligustica* was probably the founder population of HRJB. Although studies have shown that several *A. m. carnica* colonies also carried mitotype C1 (Sušnik et al., 2004), *A. m. carnica* honeybee workers usually have a black body color which is different from the mainly yellow color of HRJB honey bee workers (Our unpubl. data). Furthermore, historical records have shown that *A. m. ligustica* was first introduced into China (CNCAGR, 2011). Zhejiang Province, where HRJB originated and where it is mainly distributed, was one of the areas where beekeepers first began to raise *A. m. ligustica* in China (CNCAGR, 2011). Combining the results from our *COI-COII* sequence analysis with the morphological characters and relevant historical records, it is likely that HRJB was bred from *A. m. ligustica.*

In addition to mtDNA *COI-COII* sequences, mtDNA

ND2 sequences were also used to analyze the molecular genetic diversity of honeybees, and could provide additional useful information (Franck et al., 2000b). In our study, the results based on the *COI-COII* sequences identified the possible origin of HRJB honey bees. Furthermore, with the results based on the ND2 sequences three basic differences were found that distinguished HRJB colonies from unimproved *A. m. ligustica* colonies. Therefore, our study may provide a simple method, based on ND2 sequences, for the differentiation of HRJB from unimproved *A. m. ligustica* colonies in China. For example, discrimination between the bases C and T at position 541 of the ND2 sequence could identify 96% of the unimproved *A. m. ligustica* colonies and 93% of HRJB colonies. HRJB colonies from the Pinghu region were noted to have a larger number of mitotypes than those from the other two regions, an observation which should be considered in future conservation strategies. Pinghu, as the center of origin of HRJB, contains a large number of such colonies. The large population size, as well as the longer period of adaptation of honeybees in Pinghu, may help to maintain the high level of genetic diversity. A previous study based on microsatellites had also found that a significant genetic differentiation existed among HRJB populations from different regions (Yin et al., 2011). Although HRJB honeybees exhibit high royal jelly-producing performance, further breeding should be carried out to increase the 10-HDA (10-hydroxy-2-decenoic acid) content of royal jelly and improve the colonies' general resistance to disease (Cao et al., 2016). The heterozygosity among HRJB populations may provide favorable conditions for further selection or breeding.

To summarize, we confirmed that HRJB was probably bred from *A. m. ligustica*, with HRJB also having different genetic characteristics, based on mitochondrial DNA sequences, compared to unimproved *A. m. ligustica*. However, mtDNA can only reflect the maternal origin of HRJB. In the future, additional molecular technologies such as single nucleotide polymorphism (SNP) analysis should be applied to improve our understanding of the genetic characterization of HRJB.

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